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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/42 // C11D 3/386

A1

(11) International Publication Number: **WO 95/26398**

(43) International Publication Date: 5 October 1995 (05.10.95)

(21) International Application Number: PCT/DK95/00132

(22) International Filing Date: 27 March 1995 (27.03.95)

(30) Priority Data:
0347/94 28 March 1994 (28.03.94) DK

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

**Published**
*With international search report.*

(54) Title: A MODIFIED CELLULASE AND AN ENZYME PREPARATION COMPRISING A MODIFIED CELLULASE

(57) Abstract

Chemically modified cellulolytic enzymes (cellulases) having an improved performance and a pI which is at least one pI unit higher than that of the parent or native cellulase, e.g. which are modified by coupling an amine to the carboxyl group of glutamic acid or aspartic acid residues in the cellulase, and enzyme preparations comprising such modified cellulases are useful e.g. in the pulp and paper industry, the textile industry, for animal feed and human food and for baking purposes.

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# A MODIFIED CELLULASE AND AN ENZYME PREPARATION COMPRISING A MODIFIED CELLULASE

## FIELD OF INVENTION

The present invention relates to a chemically modified cellulase, an enzyme preparation comprising the modified cellulase and methods for using the modified cellulase and/or enzyme preparation e.g. in the pulp and paper industry, the textile industry, for animal feed and human food and for baking purposes.

## BACKGROUND OF THE INVENTION

Enzymes have been used for a long time for a variety of industrial applications. For instance important uses of enzymes are in papermaking pulp processing, in the baking industry for improving the properties of flour, in the wine and juice industries for the degradation of $\beta$-glucans, in the textile industry for bio-polishing of cellulosic fabrics, i.e. for obtaining a soft and smooth fabric by subjecting the cellulosic fabrics to treatment by cellulolytic enzymes during their manufacture, and in animal feed for improving the digestibility of vegetable protein sources.

In enzyme applications relating to cellulosic/lignocellulosic materials, the desired process conditions are quite often in the weakly acidic to alkaline range, e.g. in order to obtain a higher solubility of reaction products, or in order to avoid use of acid and thus minimize the corrosiveness of the reaction mixture. When enzymes having relatively acidic pI, e.g. below 5, are used in such "neutral to alkaline processes" the overall performance of the enzymes may be limited under the process conditions. Some enzymes at least might be expected to show an improved performance if the pI of the enzymes is shifted to a value approximating that of the pH during

application.

These considerations may apply to the use of cellulases in enzymatic processes in e.g. one or more of the industries mentioned above.

E.g. when processing papermaking pulps, the lignocellulosic fibers may be subjected to enzymatic hydrolysis by cellulolytic enzymes. Cellulolytic enzyme preparations for fibre modification may besides cellulases also contain hemicellulases, for efficient degradation of carbohydrate material in the fibre walls or in fibre debris.

Today, it is well established that the overall performance of cellulolytic enzymes on solid lignocellulosic surfaces is limited due to electrostatic repulsion forces restricting the access for the enzymes to the substrate. So far no economical or technically feasible method for overcoming this disadvantageous electrostatic repulsion has been suggested. In WO 93/11296 and WO 93/07332 it is described how the repulsion can be reduced by enzymatic removal of negatively charged glucoronic acid in the fibre matrix or by exchanging the counter ions on the acid groups in the fibre. These procedures are, however, very costly since bulk mass of lignocellulosic fibers must be treated with expensive specialty enzymes or metal salts. The latter may also cause accumulation problems in internal water recirculation system of lignocellulosic fibre processing installations.

Furthermore, up till now it has been believed that the size of the enzyme molecules is another determining parameter for the effect of enzymes acting on lignocellulosic fibers. Average fibre pore sizes have been claimed to be of the same magnitude as the average diameter of the single enzyme molecules, why in order to have access to substrate inside porous

lignocellulosics, the enzyme should be as small as possible. (Viikari, L., Kantelinen, A., Rättö, M. & Sundquist, J. (1991), Enzymes in Biomass Conversion, Chpt.2: Enzymes in Pulp and Paper Processing, p. 14, (Leatham, G.F. & Himmel, M.E., eds.).

Thus, it is still an unsolved problem how to improve the effect of enzymatic hydrolysis of lignocellulosic fibers.

## DESCRIPTION OF THE INVENTION

Surprisingly, it has been found that when cellulases are modified in a way that the negatively charged side groups are substituted or replaced, this may lead to unexpected high increases in enzyme activity and/or in substrate availability. This is despite the fact that such a modification may increase the size of the enzyme molecules.

Thus, the electrostatic repulsion may be reduced through modification of the enzyme molecules in stead of modifying the substrate. Calculated in mass quantity, the amount of substrate is typically at least 100 times more than the mass of the enzyme product used in enzymatic processes, e.g. for treatment of the lignocellulosic fibers. Accordingly, it is much more economical to modify the enzyme instead of the lignocellulosic fibers.

The present invention relates to a modified cellulase having a pI which is as least one pI unit higher than that of the parent or native cellulase, the modification being obtained by chemical modification.

The modified cellulase of the invention is believed to have an improved performance due to an increased pI value and/or an increased surface activity.

The present invention further relates to an enzyme preparation comprising one or more modified cellulases. It is obvious that the enzyme preparation of the invention may contain one or more modified cellulase components (e.g. endo-β-1,4-glucanases and cellobiohydrolases), either alone or in combination with other enzymes which have or have not been subjected to a chemical modification or an amino acid substitution with the purpose of obtaining an increased pI value and/or an increased surface activity.

In the present context, the term "improved performance" is intended to indicate that the modified enzyme, when subjected to the same standard test conditions as the parent enzyme, exhibits an improved effect compared to the parent enzyme. For enzymes intended to be used in processing of fabrics or papermaking pulp, the performance of a modified cellulase preparation is evaluated from the amount of soluble hydrolysis products, e.g. short chained carbohydrates such as cello-oligomers which are dissolved from the pulp during a treatment with said cellulase preparation minus the amount that is dissolved in a control treatment without addition of cellulase. The dissolved carbohydrates may be determined by analysis with anion exchange chromatography with pulsed amperometric detection (Dionex Corporation, Sunnyvale, CA, USA : Technical Note 20, 1989) or with the orcinol total reducing sugar method (see *Vasseur, E., Acta Chem. Scand., "A spectrophotometric study on the orcinol reaction with carbohydrates," 2:693 (1948)*). The dry matter weight loss compared to a control is often also used as an additional measure of cellulase performance.

The isoelectric point, pI, is defined as the pH value at which the enzyme molecule is neutral, i. e. the sum of electrostatic charges (net electrostatic charge) is equal to zero. In this sum of course consideration of the positive or negative nature

of the individual electrostatic charges must be taken into account. The pI may conveniently be determined experimentally by isoelectric focusing or by titrating a solution containing the enzyme.

According to the invention, the pI of the modified enzyme should preferably be at least one pI unit, more preferably at least two pI units, most preferably at least three pI units, higher than that of the parent enzyme. For example, a (parent) cellulase having a pI of 5.5 may be chemically modified as described herein resulting in a modified cellulase having a pI of 9.

In the present specification and claims, the terms "cellulase" and "cellulase component" are intended to mean an enzyme that hydrolyses cellulose. The cellulase or cellulase component may be a component occurring in a cellulase system produced by a given microorganism, such a cellulase system mostly comprising several different cellulase enzyme components including those usually identified as e.g. cellobiohydrolases, endo-β-1,4-glucanases, β-glucosidases. Alternatively, the cellulase component may be a single component, i.e. a component essentially free of other cellulase components usually occurring in a cellulase system produced by a given microorganism, the single component being a recombinant component, i.e. produced by cloning of a DNA sequence encoding the single component and subsequent cell transformed with the DNA sequence and expressed in a host. The host is preferably a heterologous host, but the host may under certain conditions also be the homologous host.

The native or unmodified cellulase or cellulase component may be derived from microorganisms which are known to be capable of producing cellulolytic enzymes, e.g. species of Humicola, Thermomyces, Bacillus, Trichoderma, Fusarium, Myceliophthora,

Phanerochaete, Schizophyllum, Penicillium, Aspergillus, and Geotricum. The derived component may be either homologous or heterologous component. Preferably, the component is homologous. However, a heterologous component which is immunologically reactive with an antibody raised against a highly purified cellulase component and which is derived from a specific microorganism is also preferred.

A preferred example of a native or parent or unmodified cellulase component is an endoglucanase component which is immunologically reactive with an antibody raised against a highly purified ~43kD endoglucanase derived from *Humicola insolens*, DSM 1800, or which is a derivative of the ~43kD endoglucanase exhibiting cellulase activity. A preferred endoglucanase component has the amino acid sequence disclosed in PCT Patent Application No. WO91/17243, SEQ ID#2, which is hereby incorporated by reference. Another preferred endoglucanase component is the core enzyme corresponding to the amino acid sequence disclosed in PCT Patent Application No. WO91/17243, SEQ ID#2, but having the amino acid sequence corresponding to position 1-213, i.e. truncated at position 213.

Yet another preferred endoglucanase component is an endoglucanase component which is immunologically reactive with an antibody raised against a highly purified ~26kD endoglucanase derived from *Aspergillus aculeatus*, CBS101.43, or which is a derivative of the ~26kD endoglucanase exhibiting cellulase activity. In the present context, the term "derived from" is intended not only to indicate an endoglucanase produced by strain CBS 101.43, but also an endoglucanase encoded by a DNA sequence isolated from strain CBS 101.43 and produced in a host organism transformed with said DNA sequence. A preferred endoglucanase component is the Endoglucanase III disclosed in International Patent Application PCT/DK93/00444, which is hereby incorporated by reference, the Endoglucanase III being

believed to exhibit a substantial homology with the sequence disclosed by Ooi et al., 1990, in "Complete nucleotide sequence of a gene coding for *Aspergillus aculeatus* cellulase (FI-CMCase)", Nucleic Acids Research, Vol. 18, No. 19: 5884.

The term "cellulosic fabric" is intended to include fabric originating from cellulose fibers, e.g. from wood pulp, and cotton. Examples of cellulosic fabrics are cotton, viscose (rayon); lyocell; all blends of viscose with other fabrics such as viscose/polyester blends, viscose/cotton blends, viscose/wool blends; flax (linen) and ramie and other fabrics based on cellulose fibers, including all blends of cellulosic fabrics with other fabrics such as wool and polyester, e.g. viscose/polyester blends, viscose/cotton blends, viscose/wool blends, viscose/cotton/polyester blends, flax/cotton blends etc.

In a preferred embodiment of the modified cellulase or the enzyme preparation of the invention, the enzyme is chemically modified by coupling an amine ligand to the carboxyl group of glutamic acid or aspartic acid residues in the enzyme. By this chemical modification, the carboxylic acid groups are neutralized, thereby increasing the pI of the enzyme. The amine ligand is preferably an aminated sugar, aminated alcohol or aminated polyalcohol. Examples of suitable aminated sugars are glucosamine, isomeric forms thereof with the general formula $C_6H_{13}O_5N$, or oligomers and polymers of the general formula $[C_6H_{11}O_4N]_n$, for example polymers of glucosamines such as chitosans. Oligomers and polymers may be either branched or linear.

If an aminated alcohol is used for coupling to the carboxyl group, it should generally contain at least 3 carbon atoms. Examples of suitable aminated alcohols are aminopropanol or aminobutanol. More preferably the amine ligand is an aminated

polyalcohol. Polyalcohols should generally contain at least 3 carbon atoms, and may for instance contain 6 carbon atoms. Examples of suitable aminated polyalcohols are glucamine, isomeric forms thereof with the general formula $C_6H_{15}O_5N$, or oligomers and polymers thereof with the general formula $[C_6H_{13}O_4N]_n$, wherein n>1.

Other suitable amine ligands are amine substituted alkanes and derivatives thereof. Preferred examples of amine substituted alkanes and their derivatives are amino acids such as lysine, polylysine; esters of amino acids; spermine; spermidine; putrescine; and the like.

The amine ligand such as an aminated sugar, alkane, alcohol or polyalcohol and polymer thereof, should have at least one amino group per monomeric unit, but should not be considered to be limited to having only one amino group per monomeric unit.

According to a preferred method, the coupling of the amine to the carboxyl group of glutamic acid or aspartic acid residues is mediated by a crosslinking agent capable of binding a carboxyl group and an amino group. The coupling reaction may suitably be carried out by standard methods as described by S.S. Wong, *Chemistry of Protein Conjugation and Cross-Linking*, CRC Press, Boca Raton, Florida, USA, 1991, in particular Chapter 2, IV, C, Chapter 4, IV and Chapter 5, II; or Wong and Wong, Enzyme Microb.Technol. 14, November 1992, pp. 866-873. A particularly preferred crosslinking agent for the coupling reaction is a carbodiimide, e.g. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).

Methods of conjugating proteins with ligands using EDC can be implemented according to manufacturer's description (e.g. Pierce Instructions 0475 C, 22980 X; 22981 X; EDC) using either the protocol for "Use of EDC for coupling of Haptens/small

ligands to carrier Proteins" or "Protocol for Efficient Two-Step coupling of Proteins in Solution Using EDC and N-hydroxysuccinimide or sulfo-N-hydroxysucciminide".

For example the enzyme may be dissolved, or transferred by dialysis or desalting by size exclusion chromatography in a coupling buffer, such as, for instance 50 mM MES pH 5.0 containing 200 mM sodium chloride. The ligand, e.g. glucosamine, may be dissolved in coupling buffer as well. The conjugation reaction may proceed by mixing enzyme and ligand to a final concentration of 3 mg/ml for both enzyme and ligand followed by mixing with 5 mg of EDC per mg of enzyme. The conjugation reaction then runs for 2 hours at room temperature with continuous stirring. The reaction is terminated by removal of surplus reagent either by desalting by size exclusion chromatography or by extensive dialysis, e.g. against 0.2 M ammonium acetate pH 6.9 at 5°C. The resulting derivative may then be stored at 5°C.

The degree of modification or incorporation of ligands may, of course, be controlled by adjustments in the initial enzyme, ligand and/or carbodiimide concentration. Variations in pH or temperature of the coupling buffer may also be included to optimise the conjugation reaction for a specific enzyme.

Naturally active site protection by substrate, substrate analogues and reversible inhibitors may be used to control of the modification reaction.

In another preferred embodiment of the enzyme preparation of the invention, the preparation comprises one or more cellulases modified by substitution of one or more amino acids as disclosed in PCT/DK93/00327 which is hereby incorporated by reference.

Preparation of modified cellulases by amino acid substitution

Several methods for introducing mutations into genes are known in the art. After a brief discussion of cloning enzyme-encoding DNA sequences, methods for generating mutations at specific sites within the enzyme-encoding sequence will be discussed.

Cloning a DNA sequence encoding a cellulase

The DNA sequence encoding a parent enzyme may be isolated from any cell or microorganism producing the enzyme in question by various methods, well known in the art. First a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the enzyme to be studied. Then, if the amino acid sequence of the enzyme is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify enzyme-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known enzyme could be used as a probe to identify enzyme-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying enzyme-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for enzyme thereby allowing clones expressing the enzyme to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., The EMBO J. 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA sequence, in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., Science 239, 1988, pp. 487-491.

Site-directed mutagenesis

Once an enzyme-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the enzyme-encoding sequence, is created in a vector carrying the enzyme gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al., (1984, Biotechnology 2:646-639). U.S. Patent number 4,760,025, by Estell et al., issued July 26, 1988, discloses the introduction of

oligonucleotides encoding multiple mutations by performing minor alterations of the cassette, however, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into enzyme-encoding DNA sequences is described in Nelson and Long, Analytical Biochemistry 180, 1989, pp. 147-151. It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Expression of modified enzymes

According to the invention, a mutated enzyme-encoding DNA sequence produced by methods described above, or any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding a modified enzyme of the invention encoding may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one

which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a cellulase variant as described herein, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amy*L), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amy*M), the promoters of the *Bacillus Amyloliquefaciens* α-amylase (*amy*Q), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the enzyme variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of

such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B.subtilis* or *B.licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

The procedures used to ligate the DNA construct of the invention encoding an enzyme variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. Molecular Cloning; A Laboratory Manual, CSH, NY, 1989).

The cell of the invention either comprising a DNA construct or an expression vector as defined above is advantageously used as a host cell in the recombinant production of an enzyme variant of the invention. The cell may be transformed with the DNA construct encoding the modified enzyme, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described below in connection with the different types of host cells.

The host cell may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are gram-positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram-negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae*, *Aspergillus awamori*, *Aspergillus aculeatus* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

The modified enzyme may be produced by cultivating a host cell as described above under conditions conducive to the production of the modified enzyme and recovering the modified enzyme from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the modified enzyme of the invention. Suitable media are available from commercial suppliers or may

be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection).

The modified cellulase secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Applications

It is contemplated that, dependent on the specificity of the modified cellulase, it may be employed for one or possibly more of the applications mentioned above, i.e. in the baking industry, in the wine and juice industry, for animal feed, and in textile and papermaking pulp processing. In a particular embodiment, the enzyme preparation of the invention may comprise a combination of one or more modified cellulases with enzymes selected from the group consisting of unmodified or modified amylases, lipases, proteases, oxidoreductases and hemicellulases.

Pulp and paper applications

In the papermaking pulp industry, the cellulase and/or enzyme preparation according to the invention may be applied advantageously e.g. as follows:

- For debarking: pretreatment with the cellulase and/or enzyme preparation according to the invention may degrade the cambium layer prior to debarking in mechanical drums resulting in

advantageous energy savings.

- For defibration: treatment of a material containing cellulosic fibers with the cellulase and/or enzyme preparation of the invention prior to refining or beating may result in reduction of the energy consumption due to the hydrolysing effect of the cellulase on the interfibre surfaces. Use of the cellulase and/or enzyme preparation of the invention may result in improved energy savings as compared to the use of unmodified enzymes, since it is believed that the modified cellulase may possess a higher ability to penetrate fibre walls.

- For fibre modification, i.e. improvement of fibre properties where partial hydrolysis across the fibre wall is needed which requires deeper penetrating enzymes (e.g. in order to make coarse fibers more flexible). Deep treatment of fibers has so far not been possible for high yield pulps e.g. mechanical pulps or mixtures of recycled pulps. This has been ascribed to the nature of the fibre wall structure that prevents the passage of enzyme molecules due to physical restriction of the pore matrix of the fibre wall. It is contemplated that the modified (i.e. derivatised) cellulases of the invention are capable of penetrating into the fibre wall.

- For drainage improvement. The drainability of papermaking pulps may be improved by treatment of the pulp with hydrolysing enzymes, e.g. cellulases. Use of the modified cellulase and/or enzyme preparation according to the invention may be more effective, e.g. result in a higher degree of loosening bundles of strongly hydrated micro-fibrils in the fines fraction (consisting of fibre debris) that limits the rate of drainage by blocking hollow spaces between fibers and in the wire mesh of the paper machine. The Canadian standard freeness (CSF) increases and the Schopper-Riegler drainage index decreases when pulp in subjected to cellulase treatment, see e.g. US

patent 4,923,565; TAPPI T227, SCAN C19:65 which are hereby incorporated by reference.

- For inter fibre bonding. Hydrolytic enzymes are applied in the manufacture of papermaking pulps for improving the inter fibre bonding. The enzymes rinse the fibre surfaces for impurities e.g. cellulosic debris, thus enhancing the area of exposed cellulose with attachment to the fibre wall, thus improving the fibre-to-fibre hydrogen binding capacity. This process is also referred to as dehornification. Paper and board produced with a cellulase containing enzyme preparation according to the invention may have an improved strength or a reduced grammage, a smoother surface and an improved printability. These improvements are believed to be a result of the improved penetrability of the modified/derivatised enzyme(s).

- For enzymatic deinking. Partial hydrolysis of recycled paper during or upon pulping by use of hydrolysing enzymes such as cellulases are known to facilitate the removal and agglomeration of ink particles. Use of a modified cellulase and/or enzyme preparation according to the invention may give a more effective loosening of ink from the surface structure due to a better penetration of the enzyme molecules into the fibrillar matrix of the fibre wall, thus softening the surface whereby ink particles are effectively loosened. The agglomeration of loosened ink particles are also improved, due to a more efficient hydrolysis of cellulosic fragments found attached to ink particles originating from the fibres.

The treatment of lignocellulosic pulp may, e.g., be performed as described in WO 91/14819, WO 91/14822, WO 92/17573 and WO 92/18688.

Textile applications

In another embodiment, the present invention relates to use of the modified cellulase and/or enzyme preparation according to the invention in the bio-polishing process. Bio-Polishing is a specific treatment of the yarn surface which improves fabric quality with respect to handle and appearance without loss of fabric wettability. The most important effects of Bio-Polishing can be characterized by less fuzz and pilling, increased gloss/luster, improved fabric handle, increased durable softness and altered water absorbency. Bio-Polishing usually takes place in the wet processing of the manufacture of knitted and woven fabrics. Wet processing comprises such steps as e.g. desizing, scouring, bleaching, washing, dying/printing and finishing. During each of these steps, the fabric is more or less subjected to mechanical action. In general, after the textiles have been knitted or woven, the fabric proceeds to a desizing stage, followed by a scouring stage, etc. Desizing is the act of removing size from textiles. Prior to weaving on mechanical looms, warp yarns are often coated with size starch or starch derivatives in order to increase their tensile strength. After weaving, the size coating must be removed before further processing the fabric in order to ensure a homogeneous and wash-proof result. It is known that in order to achieve the effects of Bio-Polishing, a combination of cellulolytic and mechanical action is required. It is also known that "super-softness" is achievable when the treatment with cellulase is combined with a conventional treatment with softening agents. It is contemplated that use of the modified cellulase and/or enzyme preparation of the invention for bio-polishing of cellulosic fabrics is advantageous, e.g. a more thorough polishing can be achieved. Bio-polishing may be obtained by applying the method described e.g. in WO 93/20278.

Stone-washing of cellulosic fabric

In yet another embodiment, the present invention relates to use of the modified cellulase and/or enzyme preparation according to the invention in "stone-washing" of dyed fabric.

"Stone-washing" of fabric is useful for obtaining a distessed "used and abused" look which in recent years has become very desirable, particularly in denim clothing. Traditionally, it involves tumbling the fabric with pumice stones while wet for a sufficient period such that the pumice abrades the fabric to produce, e.g. in the fabric panels and in the seams in case of clothing items, localized abraded areas of lighter colour. In recent years, "stone-washing" have been carried out by treating the fabric enzymatically, either in combination with pumice or without pumice or in combination with perlite, with a cellulase preparation, e.g. as described in EP-A-0 307 564, EP-A-0 435 876, and International Patent Application PCT/DK94/00360 which all describe the use of cellulolytic enzymes in a "stone-washing" process.

The modified cellulase or the enzyme preparation of the invention is most beneficially applied to cellulose-containing fabrics, such as cotton, viscose, rayon, ramie, linen, lyocell (Tencel) or mixtures thereof, or mixtures of any of these fibres. In particular, the fabric is denim. The fabric may be dyed with vat dyes such as indigo, direct dyes such as Direct Red 185, sulphur dyes such as Sulfur Green 6, or reactive dyes fixed to a binder on the fabric surface.

In a most preferred embodiment of the method of the invention, the fabric is indigo-dyed denim, including clothing items manufactured therefrom.

The amount of modified cellulase or cellulase preparation used to treat the dyed fabric typically depends on the ratio of

cellulolytic enzyme and optionally buffer and pumice or perlite in the composition and the dry weight of the dyed fabric to be washed. Typically, the composition used in the process of the invention contain a minimum of 20 ECU of endoglucanase and, in case of using perlite, a minimum of 20 w/w% of perlite to obtain the stone-washed look. In a preferred mode the dyed fabric may be contacted with about 40-150 ECU of endoglucanase per litre of washing liquor for 75 minutes at about 55°C. The preferred pH is dependent on the pH optimum of the cellulolytic enzyme, i.e. whether an acid, neutral, or alkaline cellulase is applied.

Baking

In yet another embodiment, the present invention relates to use of the modified cellulase and/or enzyme preparation in baking flour so as to improve the development, elasticity and/or stability of dough and/or the volume, crumb structure and/or anti-staling properties of the baked product. Although the enzyme preparation may be used for the preparation of dough or baked products prepared from any type of flour or meal (e.g. based on rye, barley, oat, or maize), the modified cellulase and/or enzyme preparation of the invention have been found to be particularly useful in the preparation of dough or baked products made from wheat or comprising substantial amounts of wheat. The baked products produced with the modified cellulase and/or enzyme preparation of the invention includes bread, rolls, baguettes and the like. For baking purposes the enzyme preparation of the invention may be used as having cellulase as the major enzymatic activity, or may be used in combination with other enzymes such as a lipase, an amylase, an oxidase (e.g. glucose oxidase, peroxidase), a laccase and/or a protease; the lipase, amylase, oxidase and laccase optionally being modified as described in Danish patent application no. DK

0259/94.

Beer brewing

In yet another embodiment, the present invention relates to use of a modified cellulase and/or enzyme preparation according to the invention in the beer brewing industry in particular to improve the filterability of wort e.g. containing barley and/or sorghum malt. The cellulase and/or enzyme preparation may be used in the same manner as pentosanases conventionally used for brewing, e.g. as described by Viëtor et al., 1993, J. Inst. Brew., May-June, 99, pp. 243-248, and EP 227 159. Furthermore, the modified cellulase and/or enzyme preparation of the invention may be used for treatment of brewers spent grain, i.e. residuals from beer wort production containing barley or malted barley or other cereals, so as to improve the utilization of the residuals for, e.g., animal feed.

Degradation of plant material

In yet another embodiment, the present invention relates to use of a modified cellulase and/or enzyme preparation according to the invention for degradation of plant material e.g. cell walls.

It is contemplated that the modified cellulase and/or enzyme preparation of the invention is useful in the preparation of wine, fruit or vegetable juice in order to increase yield. Cellulases according to the invention may also be applied for enzymatic hydrolysis of various plant cell-wall derived materials or waste materials, e.g. agricultural residues such as wheat-straw, corn cobs, whole corn plants, nut shells, grass, vegetable hulls, bean hulls, spent grains, sugar beet pulp, and the like. The plant material may be degraded in order to improve different kinds of processing, facilitate

purification or extraction of other components like purification of beta-glucan or beta-glucan oligomers from cereals, improve the feed value, decrease the water binding capacity, improve the degradability in waste water plants, improve the conversion of e.g. grass and corn to ensilage, etc.

Animal feed

In yet another embodiment, the present invention relates to use of a cellulase and/or enzyme preparation according to the invention in animal feed (or for the treatment of animal feed prior to ingestion by the animal). Modified cellulase and/or enzyme preparation is preferably added to the feed in an amount which is efficient for improving the digestibility of vegetable protein sources, e.g. cereals and legumes. Thus, the cellulase and/or enzyme preparation of the present invention may be used for modification of animal feed and may exert its effect either *in vitro* (by modifying components of the feed) or *in vivo*.

The following examples further illustrate the present invention, and they are not intended to be in any way limiting to the scope of the invention as claimed.

**Example 1**

**Conjugation of *Aspergillus aculeatus* endoglucanase III with glucosamine mediated by EDC**

Conjugation of an endoglucanase from *Aspergillus aculeatus*, i.e. the recombinant endoglucanase denoted Endoglucanase III as disclosed in International Patent Application PCT/DK93/00444, with glucosamine through carbodiimide mediated coupling was performed according to standard procedures.

An enzyme stock solution was prepared by dissolving approximately 100 mg/ml of endoglucanase III in water, followed by size exclusion chromatography on Superdex 75 (from Pharmacia AB, Sweden) in coupling buffer (50 mM MES pH 5.0 containing 250 mM sodium chloride). The glucosamine was dissolved in coupling buffer as well.

The conjugation reaction proceeded by mixing enzyme and glucosamine to a final concentration of about 1 mg/ml for both enzyme and glucosamine followed by addition of 5 mg of EDC per mg of enzyme to mediate the reaction. The reaction mixture also contained 200 mM of cellobiose for active site protection. The conjugation reaction continued for 1 hour at room temperature with continuous magnetic stirring.

The reaction was terminated by desalting on a Sephadex G-15 column equilibrated with 0.2 M ammonium acetate, pH 6.9, at room temperature. The derivative was stored at 5°C.

The endoglucanase-glucosamine derivative prepared according to the above described procedure was shown to be monomeric by size-exclusion chromatography on a TSK-G2000SW column, has a pI value of about 9 as determined by isoelectric focusing and 26% residual cellulase activity when compared to the parental

(native) enzyme which has a pI of 5.5. The activity was measured according to the standard Novo Nordisk cellulase methods AF-275-GB/AF-295-GB (available from Novo Nordisk A/S on request) which are hereby incorporated by reference.

**Example 2**

By means of tensiometry it was shown that glucosamination of the cellulase (endoglucanase) described in example 1 results in a significant increase in the interfacial activity of the enzyme at alkaline pH values.

The measurements were performed with a Sigma 70 tensiometer from KSV, Finland, equipped with a Wilhelmy Pt-plate.
The experiments were carried out by injecting 25µl highly purified enzyme (adjusted to $OD_{280nm}$=1.43 for both the native (parent) endoglucanase and the glucosamine derivative ("endoglucanase-GA") into a 100 ml buffer solution, while following the surface tension $\gamma$ with time.
Measurements were performed at 25°C in 50mM glycine pH10 + 500 mM NaCl. Addition of an excess of neutral salt was done in order to increase the adsorption of enzyme at the air-water interface.

With the native enzyme (the 26 kD endoglucanase) it is evident that no detectable adsorption takes place, as the surface pressure (which is a function of the extent of adsorption) remains essentially zero following the addition of the enzyme. Contrary, with the endoglucanase-GA the isoelectric point of the enzyme is approached and a significant extent of adsorption is evident from the increased surface pressure, see Figure 1.

Figure 1 illustrates the effect of glucosamination on the A/W-adsorption of cellulase by showing the surface pressure (mN/m)

as a function of the time after addition of enzyme (at the time 2 min; 50 mM glycin/500 mM NaCl; temperature 298K). The bold line illustrates the effect of glucosaminated cellulase and the skip line illustrates the zero effect of the native cellulase.

The surface pressure is defined as -Δsurface tension when the surface tension of the pure buffer solution and the enzyme containing buffer solution, respectively, is compared.

**Example 3**

**Endoglucanase treatment of oxygen bleached kraft pulp**

Treatment of kraft pulp with endoglucanase is applied e.g. in enzymatic deinking of office waste paper or for improving interfibre bonding in kraft liner. The example demonstrates how the activity of a cellulase, i.e. an endoglucanase, may be increased by modification according to the invention.

The purified *Aspergillus aculeatus* endo-1,4-β-glucanase described in example 1 was derivatised according to example 1.

An industrial oxygen bleached kraft pulp obtained from hardwood and an oxygen bleached pulp obtained from softwood was treated with a the purified endoglucanase at a dose rate of 5000 ECU per kg oven dry pulp. (The ECU assay quantifies the amount of catalytic activity present in the sample by measuring the ability of the sample to reduce the viscosity of a solution of carboxy-methylcellulose (CMC). The assay is carried out at 40°C, pH 7.5 using a relative enzyme standard for reducing the viscosity of the CMC substrate and the resulting reduction in viscosity may be determined by a vibration viscosimeter (e.g. MIVI 3000 from Sofraser, France). Determination of the cellulolytic activity, measured in terms of ECU, may be

determined according to the analysis method AF 301.1 which is available from the Applicant upon request.)

First the pulp was dispersed at 1.5% consistency in a laboratory pulper with 10.000 revolutions according to SCAN C18 and dewatered. Then the pH was adjusted with sulphuric acid. The samples were diluted to 10% consistency and endo-glucanase was added. The pulp samples were now incubated 3 hours in closed plastic bags immersed in thermostated water at 50°C. The bags were kneaded by hand 30 seconds every 15 minutes. After the incubation time the pulp samples were drained. Samples of the water phase were filtered through a 45 micrometer filter in order to remove any debris from the pulp, and the final pH was measured. The water samples were analysed for dissolved total carbohydrate according to the orcinol method. The results are shown in Table 1 and 2 below. From the results it is concluded that the derivatised endo-glucanase performs significantly better than the unmodified (native) endoglucanase (the reference) on both hardwood and softwood pulps. The benefit is most pronounced at the high pH.

Table 1: Results from treatment of oxygen bleached hardwood kraft pulp at pH 5.2 and 6. The concentration of dissolved glucose is a measure of the performance of the cellulase. The reference has been treated with the unmodified parent endo-glucanase in a dosage equal to what is applied for the derivatised endoglucanase.

Table 1

| O2 HW kraft | Final pH | glucose g/kg pulp |
|---|---|---|
| Reference pH 5.2 | 5.15 | 2.05 |
| Derivate pH 5.2 | 5.18 | 2.32 |
| Reference pH 6.0 | 6.00 | 1.25 |
| Derivate pH 6.0 | 5.99 | 2.26 |

Table 2: As Table 1 but the substrate is oxygen bleached softwood kraft pulp.

Table 2

| O2 SW kraft | Final pH | glucose g/kg pulp |
|---|---|---|
| Reference pH 5.1 | 5.10 | 1.31 |
| Derivate pH 5.1 | 5.11 | 1.81 |
| Reference pH 6.1 | 6.11 | 0.87 |
| Derivate pH 6.1 | 6.11 | 1.58 |

**Example 4**

**Endoglucanase treatment of cotton**

Cotton materials may be treated with cellulolytic enzymes e.g. to create a smoother fibre surface or to remove impurities from the surface.

In this experiment, cotton fluff and woven fabric was treated

similarly to the kraft pulp samples as described in example 3 except for the repulping step, that was omitted. The woven fabric was treated in a Launder-Ometer.

The concentration of dissolved glucose as measured by the orcinol method was used as a measure of the effect of the treatment. The results are shown in Table 3 and 4 below. The results show that derivatisation causes more than a doubling of the effect on cotton fluff at both pH = 5.0 and pH = 5.9.

For cotton fabrics the increase in performance is approximately 40% at pH 5, but 70% at pH 6, supporting that the derivatisation is most advantageous at higher pH.

Table 3: Results from treatment of cotton fluff at pH 5.0 and 5.9. The concentration of dissolved glucose is a measure of the performance of the cellulase. The reference has been treated with the unmodified parent endo-glucanase in a dosage equal to what is applied for the derivatized endo-glucanase.

Table 3

| Cotton fluff | Final pH | glucose g/kg pulp |
|---|---|---|
| Reference pH 5.0 | 4.96 | 0.71 |
| Derivate pH 5.0 | 5.00 | 1.57 |
| Reference pH 5.9 | 5.90 | 0.67 |
| Derivate pH 5.9 | 5.91 | 1.48 |

Table 4: As Table 3 but the substrate is cotton fabrics.

Table 4

| Cotton fabrics | Final pH | glucose g/kg pulp |
|---|---|---|
| Reference pH 5.0 | 5.03 | 1.66 |
| Derivate pH 5.0 | 5.08 | 2.30 |
| Reference pH 6.0 | 5.95 | 1.12 |
| Derivate pH 6.0 | 5.97 | 1.93 |

**Example 5**

A commercial oxygen bleached kraft pulp made from Scandinavian softwood was treated 3 hours at 10% consistency with cellulase preparations. The treatments were carried out at pH 7 and 8.8, at 55°C in a 0.025 M phosphate buffer. After the treatment time a sample of the water phase was collected and the reaction was stopped by washing the pulp with boiling water. The enzymes were dosed on an equal activity basis, the dosage was 4000 ECU/kg pulp. The final content of total glucose as determined by the orcinol method, is listed in the table. As can be seen derivation enhances the performance at high pH.

| g glucose/kg pulp | pH 7.0 | pH 8.8 |
|---|---|---|
| A (prior art) | 0.74 | 0.25 |
| B (invention) | 0.29 | 0.46 |

The cellulase preparations A and B, respectively, used in this example were highly purified 43kD endoglucanase derived from *Humicola insolens*, DSM 1800, "B" being a modified endoglucanase

of the invention prepared as described in Example 1. The cellulolytic activity of the preparations were:

A: 4700 ECU/ml
B: 226.5 ECU/ml

CLAIMS

1. A modified cellulase having an improved performance and a pI which is as least one pI unit higher than that of the parent or native cellulase, wherein the modification is obtained by chemical modification.

2. A modified cellulase according to claim 1, wherein an amine is coupled to the carboxyl group of glutamic acid or aspartic acid residues in the cellulase.

3. A modified cellulase according to claim 2, wherein the amine is an aminated sugar, aminated alkane, aminated alcohol, aminated polyalcohol or amino acid or an ester or other derivatives thereof.

4. A modified cellulase according to claim 3, wherein the aminated sugar is glucosamine, isomeric forms thereof, or oligomers or polymers thereof.

5. A modified cellulase according to claim 3, wherein the aminated alcohol having at least 3 carbon atoms, for instance aminopropanol or aminobutanol.

6. A modified cellulase according to claim 3, wherein the aminated polyalcohol is D-glucamine, isomers thereof, or oligomers or polymers thereof.

7. A modified cellulase according to claim 3, wherein the amino acid is lysine, spermine, spermidine, putrescine, or polymers thereof such as polylysine.

8. A modified cellulase according to claim 2, wherein the coupling of the amine to the carboxyl group of glutamic acid or aspartic acid residues is mediated by a crosslinking agent

capable of binding a carboxyl group and an amino group.

9. A modified cellulase according to claim 8, wherein the crosslinking agent is selected from the group consisting of carbodiimides, isoxazolium derivatives, chloroformates or carbonyldiimidazole.

10. A modified cellulase according to claim 9, wherein the crosslinking agent is a carbodiimide.

11. A modified cellulase according to any of the claims 1-10, wherein the pI of the modified enzyme is as least two pI units higher than the pI of the parent or native cellulase.

12. A modified cellulase according to any of the claims 1-10, wherein the pI of the modified enzyme is as least two pI units higher than the pI of the parent or native cellulase.

13. An enzyme preparation comprising a modified cellulase according to any of the claims 1-10 and one or more enzymes selected from the group consisting of amylases, lipases, cellulases, hemi-cellulases, pectinases, peroxidases and laccases.

14. A method for the treatment of lignocellulosic fibers, wherein the fibers are treated with a modified cellulase according to any of the claims 1-12 or an enzyme preparation according to claim 13 in an amount which is efficient for improving the fibre properties.

15. A method according to claim 14, wherein the enzyme preparation comprises an endo-$\beta$-1,4-glucanase.

16. A method according to claim 14, wherein the enzyme preparation comprises a cellobiohydrolase.

17. A method according to any of the claims 14-16, wherein the lignocellulosic fibers are kraft pulp which are treated with the modified cellulase or the enzyme preparation in an amount which is efficient for substantially improving the drainability, i.e. enhancing the Canadian Standard Freeness (CSF) or reducing the Schopper-Riegler drainage index.

18. A method according to any of the claims 14-16 for enzymatic deinking of recycled paper pulp, wherein the modified cellulase or the enzyme preparation is applied in an amount which is efficient for effective removal of ink from the fibers.

19. A method of improving the properties of baking flour, wherein a modified cellulase according to any of the claims 1-12 or an enzyme preparation according to claim 13 is added to the baking flour in an amount which is efficient for improving the properties of the flour.

20. A method of improving the digestibility of a vegetable protein source, wherein animal feed or human food of essentially vegetable origin is treated with a modified cellulase according to any of the claims 1-12 or an enzyme preparation according to claim 13 in an amount which is efficient for improving the digestibility of the vegetable protein source.

21. A method of obtaining a soft and smooth cellulosic fabric, wherein the fabric is treated with a modified cellulase according to any of the claims 1-12 or an enzyme preparation according to claim 13 in an amount which is efficient for obtaining a soft and smooth fabric.

22. A method for the treatment of dyed fabric, preferably cellulosic fabric, more preferably denim, the method comprising contacting, in an aqueous medium, the fabric with a modified

cellulase according to any of the claims 1-12 or an enzyme preparation according to claim 13 in an amount effective for providing localised variation in the colour density of the surface of dyed fabric.

23. A method according to claim 21 or 22, wherein the modified cellulase is a modified endo-β-1,4-glucanase, and the enzyme preparation comprises a modified endo-β-1,4-glucanase.

24. A method according to claim 21 or 22, wherein the modified cellulase is a modified cellobiohydrolase, and the enzyme preparation comprises a modified cellobiohydrolase.

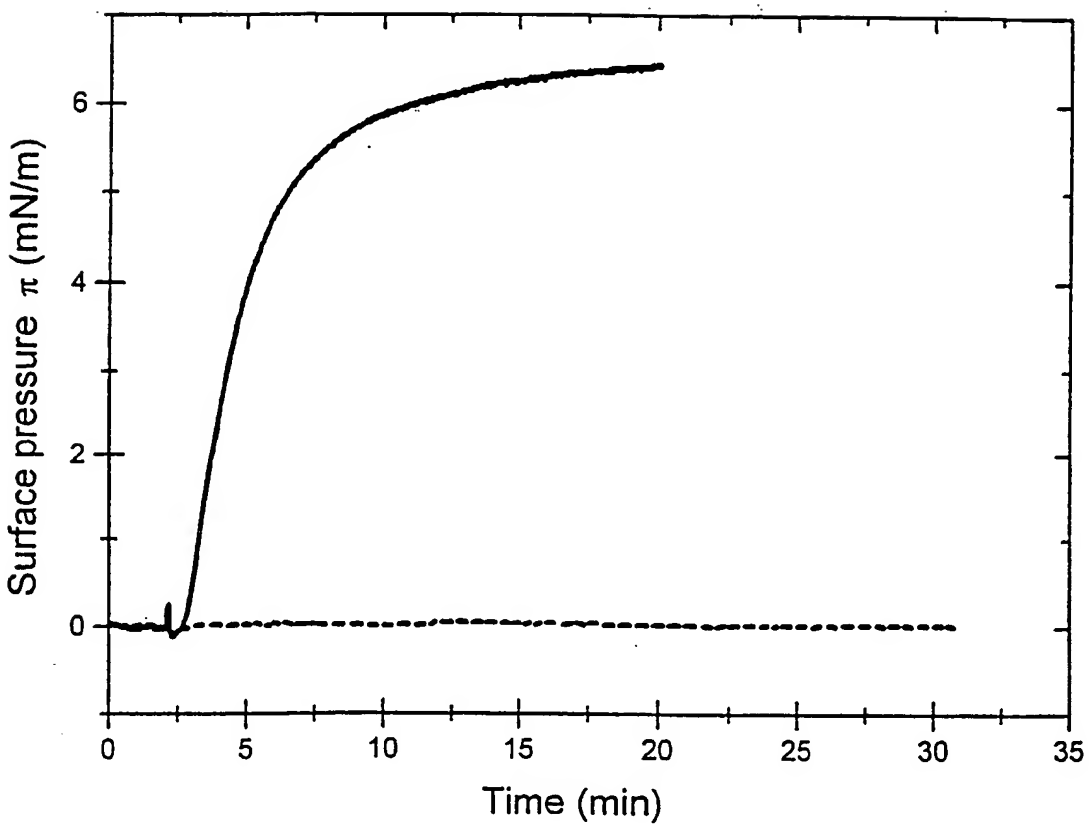


Fig. 1

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00132

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/42 // C11D 3/386
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL, MEDLINE, EMBASE, BIOSIS

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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"B" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 6 July 1995 | 10 -07- 1995 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br>Carolina Palmcrantz<br>Telephone No. +46 8 782 25 00 |

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International application No.
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